

Binding of Cysteine Synthase to the STAS Domain of Sulfate Transporter and Its Regulatory Consequences*[§]

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The sulfate ion (SO_4^{2-}) is transported into plant root cells by SO_4^{2-} transporters and then mostly reduced to sulfide (S^{2-}). The S^{2-} is then bonded to *O*-acetylserine through the activity of cysteine synthase (*O*-acetylserine (thiol)lyase or OASTL) to form cysteine, the first organic molecule of the SO_4^{2-} assimilation pathway. Here, we show that a root plasma membrane SO_4^{2-} transporter of *Arabidopsis*, SULTR1;2, physically interacts with OASTL. The interaction was initially demonstrated using a yeast two-hybrid system and corroborated by both *in vivo* and *in vitro* binding assays. The domain of SULTR1;2 shown to be important for association with OASTL is called the STAS domain. This domain is at the C terminus of the transporter and extends from the plasma membrane into the cytoplasm. The functional relevance of the OASTL-STAS interaction was investigated using yeast mutant cells devoid of endogenous SO_4^{2-} uptake activity but co-expressing SULTR1;2 and OASTL. The analysis of SO_4^{2-} transport in these cells suggests that the binding of OASTL to the STAS domain in this heterologous system negatively impacts transporter activity. In contrast, the activity of purified OASTL measured *in vitro* was enhanced by co-incubation with the STAS domain of SULTR1;2 but not with the analogous domain of the SO_4^{2-} transporter isoform SULTR1;1, even though the SULTR1;1 STAS peptide also interacts with OASTL based on the yeast two-hybrid system and *in vitro* binding assays. These observations suggest a regulatory model in which interactions between SULTR1;2 and OASTL coordinate internalization of SO_4^{2-} with the energetic/metabolic state of plant root cells.

Most environmental sulfur exists in the oxidized form, primarily SO_4^{2-} , which is reduced and assimilated by plants. Assimilation of exogenous SO_4^{2-} by plants involves its transport into cells through a $\text{H}^+/\text{SO}_4^{2-}$ symporter, reduction of internalized SO_4^{2-} to sulfide (S^{2-}), and then the covalent bonding of S^{2-} to *O*-acetylserine (OAS)² to form cysteine.

Because SO_4^{2-} uptake consumes energy and the cells have a limited ability to store sulfur-containing compounds, the uptake and assimilation of SO_4^{2-} must be coordinated with cell growth and metabolic activities and would likely be responsive to rapid changes in environmental conditions. However, regulatory mechanisms that modulate the activity of SO_4^{2-} uptake over short time scales are not known. Over longer periods of time, specific regulatory mechanisms control the transcription of SO_4^{2-} transporter genes (1–3), and other mechanisms appear to function at the post-transcriptional level (4). *Arabidopsis thaliana* has two high affinity, root-localized SO_4^{2-} transporters, SULTR1;1 and SULTR1;2, that take up SO_4^{2-} from the rhizosphere. *SULTR1;2* transcript levels are high in sulfur-rich conditions and increase slightly upon sulfur deprivation, whereas the *SULTR1;1* transcript levels increase by >100-fold during sulfur deprivation (1, 5, 6). The SO_4^{2-} transporters (SLC26A family) are structurally conserved with a large N-terminal catalytic domain, an ~30 amino acid linking region (L), and a C-terminal, cytosolic STAS domain (7). The SULTR1;2 STAS domain is essential for proper transporter localization and influences its stability and catalytic characteristics (8, 9). This domain, present on SLC26A family transporters in plants and animals, likely interacts with other proteins as it has significant similarity to the *Bacillus subtilis* anti- σ -factor antagonist SpoIIAA, which binds SpoIIAB to regulate σ -factor activity (10). In fact, the STAS domain of SLC26A3, a chloride/bicarbonate exchanger, interacts with the regulatory domain of the cystic fibrosis transmembrane conductance regulator to control bicarbonate secretion by epithelial cells (11).

Based on the findings discussed above, we hypothesized that SULTR1;2 activity may be modulated by interactions of its STAS domain with other proteins. To identify potential SULTR1;2 partner proteins, we performed a yeast two-hybrid screen of an *A. thaliana* cDNA library using the SULTR1;2 LSTAS domain as bait. A cDNA identified in the screen encodes an isoform of cysteine synthase (*O*-acetylserine (thiol)lyase or OASTL) that is expressed in the cytosol of root cortex cells (12); like this OASTL isoform, SULTR1;2 is also expressed in root cortex cells where SO_4^{2-} is taken up from the soil solution (6). The interaction between SULTR1;2 and OASTL was further demonstrated by various *in vivo* and *in vitro* assays and was shown to affect the activities of both partner proteins. Our results are discussed in the context of interactions among component proteins involved in SO_4^{2-} acquisition and assimilation, and we introduce a model that suggests a potential role of this interaction in controlling the sulfur homeostasis in roots.

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[§] The on-line version of this article (available at <http://www.jbc.org>) contains supplemental Table 1 and Figs. 1 and 2.

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² The abbreviations used are: OAS, *O*-acetylserine; OASTL, *O*-acetylserine (thiol)lyase; MBP, maltose-binding protein; Ni-NTA, nickel-nitrilotriacetic acid; BiFC, bimolecular fluorescence complementation; L, linking region; SAT, serine acetyltransferase; MOPSO, 2-hydroxy-3-morpholinopropane-sulfonic acid.

EXPERIMENTAL PROCEDURES

Yeast Strains—The strains of *Saccharomyces cerevisiae* used in this study were EGY48 (*MAT α* , *his3*, *trp1*, *ura3*, *6LexAop::LEU2*) for the yeast two-hybrid screen (13) and CP154-7B (*MAT α* , *ade2*, *his3*, *leu2*, *trp1*, *ura3*, *sul1::LEU2*, *sul2::URA3*), which is null for both *Sul1* and *Sul2*, the endogenous genes encoding SO_4^{2-} transporters (14). Yeast cells were transformed using the lithium acetate method (15).

Yeast Two-hybrid System—We used gene fragments to synthesize the SULTR1;2 STAS domain, with or without the linker region (9); these peptide domains were designated 1;2LSTAS (Linker plus STAS, from Ser-490 to Val-653, the last amino acid in the protein) or 1;2STAS (STAS without Linker, from Ile-520 to Val-653). A 10-alanine adaptor was encoded by the 5' end of the forward primer to separate the STAS and LSTAS from fusion partner polypeptides. The amplified product was ligated into bait and prey vectors, pEG202 and pJG4-5, respectively. Analogous constructs were prepared using sequences encoding the corresponding LSTAS and STAS regions of SULTR1;1. OASTL gene fragments of various lengths were amplified and cloned into the pJG4-5 prey vector. Appropriate combinations of bait and prey constructs were transformed into EGY48 harboring a LacZ reporter plasmid (pSH18-34), and *S. cerevisiae* transformants were selected on medium lacking uracil, histidine, and tryptophan. Protein expression from both the bait and prey constructs was confirmed by Western blot analysis using LexA antibodies (Invitrogen) to detect expression from the bait constructs and hemagglutinin antibodies (Roche Applied Science) to detect expression from the prey constructs. β -Galactosidase assays were performed according to standard procedures. All of the oligonucleotides used as primers in this study are listed in supplemental Table 1. The LSTAS domain of SULTR1;2 in the bait vector (pEG202) was used to screen for interacting proteins encoded by an *A. thaliana* cDNA library inserted into the prey vector (pJG4-5).

Protein Purification—The OASTL coding region was cloned into pET28a (Novagen, Gibbstown, NJ) to generate a C-terminally His₆-tagged OASTL protein (OASTL-His₆) or N-terminally His₆-tagged protein (His₆-OASTL). The SULTR1;2 or SULTR1;1 STAS domains, with or without the L regions, were cloned into the pMalc2 \times vector (PerkinElmer Life Sciences), generating MBP-STAS or MBP-LSTAS. Recombinant proteins were overexpressed in BL21(DE3)pLysS and purified using Ni-NTA (Qiagen, Valencia, CA) or amylose resin (PerkinElmer Life Sciences). Two mutations (K46A and H221A) were separately introduced into the OASTL cDNA using PCR, and the final plasmid constructs were prepared in a similar way as described for the construct with the unaltered OASTL coding region.

Preparation of Microsomal Fraction Containing Overexpressed SULTR1;2-4MYC—To test *in vitro* binding of SULTR1;2-4MYC to His₆-OASTL, the SULTR1;2 cDNA was cloned into pGWB16 (16), and the resulting plasmid was introduced into *Agrobacterium tumefaciens*. *A. tumefaciens* carrying this plasmid was inoculated into cells on the abaxial side of *Nicotiana benthamiana* leaves as described previously (17). Five days after the inoculation, leaves were harvested by emersion and frag-

mentation in liquid nitrogen and homogenized into a powder in ice-cold buffer (250 mM sucrose, 100 mM HEPES/KOH (pH 7.5), 15 mM EGTA, 5% glycerol, 0.5% polyvinylpyrrolidone, 1 mM phenylmethylsulfonyl fluoride). After removing cell debris by centrifugation at 1,600 \times g for 10 min and filtration through Miracloth, the microsomal fraction was collected by centrifuging the suspension for 1 h at 10,000 \times g and resuspending the pellet in the interaction buffer (50 mM NaH₂PO₄/Na₂HPO₄, 100 mM NaCl, 5% glycerol, 1% Triton X-100, and 10 mM imidazole). After solubilizing the microsomal fraction for 1 h on ice, cell debris was precipitated by centrifugation for 20 min at 20,000 \times g.

In Vitro Binding—Potential binding proteins were mixed in a 1:1 ratio and incubated in 1 \times phosphate-buffered saline (pH 7.4) for 1 h at 22 $^{\circ}$ C. The amylose resin was added to bind the MBP-fused proteins along with interacting proteins. To test *in vitro* binding of SULTR1;2-4MYC to His₆-OASTL, a microsomal fraction containing overexpressed SULTR1;2-4MYC was prepared as described above. His₆-OASTL was added and incubated at 4 $^{\circ}$ C for 2 h with continuous rotation. Ni-NTA was added to bind His₆-fused proteins, which would also capture interacting proteins.

BiFC—cDNAs were cloned in BiFC vectors using gateway technology (17). *A. tumefaciens* gv3101 carrying a plasmid encoding the p19 protein (18) expressing the binding pairs were co-inoculated into the apoplastic space surrounding epidermal cells of *N. benthamiana*. Fluorescence was monitored 5 days after inoculation by confocal microscopy. A piece of infected leaf was mounted onto slides, and fluorescence was imaged using a Nikon TMD200 inverted fluorescence microscope equipped with a Nikon 60 \times 1.2-numerical aperture, a water immersion objective, and a Bio-Rad MRC 1024 confocal head. All images are projection stacks of 25 confocal sections (0.5 μ m each).

SO_4^{2-} Uptake—*S. cerevisiae* CP154-7B cells harboring plasmids expressing SULTR1;2-HA (8) and OASTL-FLAG (or an empty vector) were used to determine SO_4^{2-} uptake rates as described previously (8), with a minor modification. Cells were grown in SD medium containing 500 μ M SO_4^{2-} without Met, His, or Trp (supplemented with 2% glucose, 1.3 mM OAS, and 4 mM Ser) to $A_{600} = 0.2$. The cells were then pelleted by centrifugation (5 min at 1300 \times g) and resuspended in either SO_4^{2-} -rich medium containing 1 mM SO_4^{2-} or SO_4^{2-} -poor medium containing 10 μ M SO_4^{2-} . After a 2.5-h incubation in fresh medium, the cells were harvested and washed in a buffer containing 15 mM NH₄Cl, 6.6 mM KH₂PO₄, 0.5 mM K₂HPO₄, 1.7 mM NaCl, 0.7 mM CaCl₂, 2 mM MgCl₂ (pH 5.8). Cells were kept on ice from the time of harvest to the time of assay. The cell suspension was supplemented with 2% glucose (final concentration) and maintained at 30 $^{\circ}$ C for 4 min prior to the initiation of the SO_4^{2-} uptake assay, as described previously (8). Assays were performed independently at least five times (five biological replicates) for each value and CP154-7B cells were freshly transformed with the appropriate combination of plasmid DNAs every time uptake assays were performed; independently transformed cell lines were also tested at least five times for all the data shown in Fig. 4B.

Interaction of Sulfate Transporter with Cysteine Synthase

OASTL-FLAG Construct Preparation—OASTL-FLAG was expressed from a *PMA1* promoter in a plasmid derived from pJG4-5 (PerkinElmer Life Sciences). The *PMA1* promoter, OASTL-FLAG coding sequence, and *ADHI* terminator were introduced into pJG4-5 at *SacI*-*Bam*HI, *Bam*HI-*Sall*, and *Sall*-*NotI* restriction sites, respectively.

OASTL Activity Assay—The enzymatic activity of OASTL-His₆ was assayed as described previously (19), based on established protocols (20, 21). OASTL-His₆ was added to a final concentration of 1 ng/μl to 100 mM MOPSO buffer (pH 7.0), 10 mM dithiothreitol. The MBP-STAS polypeptide was added at a 1-, 7-, and 12-fold molar concentration relative to that of OASTL. This reaction mixture (final vol. 250 μl) was incubated at 22 °C for 30 min with gentle rotation, followed by the addition of 250 μl of a solution containing 100 mM MOPSO (pH 7.0), 1 mM Na₂S, and 10 mM OAS to initiate the OASTL reaction; 100-μl samples were withdrawn from the reaction after 10 s and 6 and 12 min and mixed with 20 μl of ice-cold 50% trichloroacetic acid. The acidified reaction mixture was maintained on ice for at least 10 min, and precipitated proteins were pelleted by microcentrifugation at maximal speed. 100 μl of the supernatant was added to 100 μl of 2.5% ninhydrin solution, and the mixture was heated at 95 °C for 5 min. After a brief period of cooling on ice, 200 μl of ethanol was added to the solution, and the A₅₆₂ was determined. The specific activity of OASTL-His₆ protein in the absence of MBP-STAS (the mock treatment) was 8.2 ± 3.0 μmol/min/mg (micromoles of cysteine molecules synthesized per min by 1 mg of OASTL-His₆ protein, *n* = 12) in a reaction mixture containing 1 mM Na₂S and 10 mM OAS.

RESULTS

Yeast Two-hybrid Screen Reveals Interaction between the STAS Domain of SULTR1;2 and OASTL—To elucidate interactions of the STAS domain of SO₄²⁻ transporters with other proteins in the cell, we initially focused our studies on the SULTR1;2 STAS domain in a yeast two-hybrid analysis. We first generated a bait construct with the LexA DNA binding domain followed by a 10-alanine spacer sequence that was fused to the L-STAS domain of SULTR1;2. This bait construct was used to screen a cDNA library constructed from mRNA isolated from whole *A. thaliana* seedlings. One cDNA identified in the screen encoded a cytoplasmic isoform of OASTL (At4g14880, OASA1 (22), cytACS1 (23), or Atcys-3A (12)) with a 110-amino acid deletion at its N terminus; this truncated protein is designated OASTL111A because the amino acid at position 111 (the first amino acid of the truncated protein) is an alanine. The identical cDNA encoding the truncated OASTL was also generated from reverse transcription of OASTL mRNA from *A. thaliana* ecotype Col-0; this cDNA was used in the experiments described below. Fig. 1 shows interactions between the SULTR1;2 STAS domain, with and without the L sequence (1;2LSTAS and 1;2STAS), and full-length and various truncated forms of OASTL, tested in the yeast two-hybrid system. The 1;2LSTAS interacts with OASTL39M, -111A, and -144M but not with -1M (full-length OASTL) based on both β-galactosidase activity and leucine auxotrophy assays (Fig. 1A). A negative result for the interaction between OASTL1M and 1;2LSTAS is likely a consequence of constraints

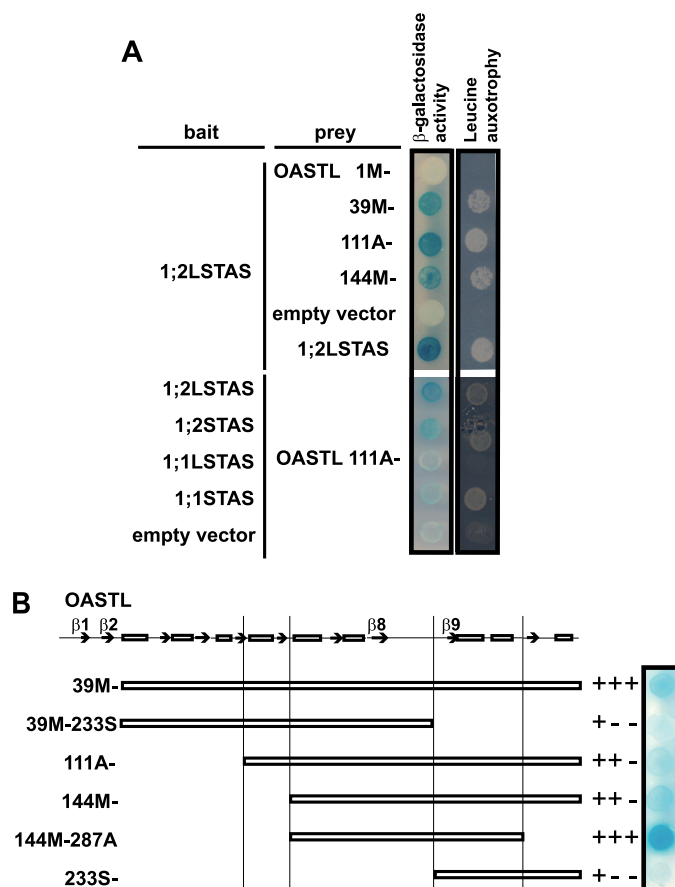


FIGURE 1. Use of yeast two-hybrid system to test interactions between LSTAS and OASTL by β-galactosidase activity and leucine auxotrophic growth assays. A, LSTAS from SULTR1;2 (1;2LSTAS) or SULTR1;1 (1;1LSTAS) were N-terminally fused to LexA and used as bait. Various lengths of the OASTL (1M, 39M, 111A, and 144A, where 1M represent full-length OASTL and the others are N-terminal truncations to amino acids 39, 111, and 144, respectively) were fused to the C terminus of the activation domain and used as prey. B, determination of the domain of OASTL that interacts with SULTR1;2 STAS in yeast two-hybrid system by β-galactosidase activity. The various truncated OASTL polypeptides were tested for their ability to bind 1;2 LSTAS. The top line is a representation of the secondary structure of OASTL (19); the rectangles represent α-helices, and the arrows represent β-strands. Number of + indicate the strengths of β-galactosidase activity indicating the strengths of interaction. Images are from representative assay plates.

of the system with respect to structure and/or regulation in the heterologous system; additional *in vitro* and *in vivo* binding assays, shown below, yield positive results with full-length OASTL. The 1;2STAS domain without the L region also interacts with OASTL111A, but this interaction is somewhat weaker than that of 1;2LSTAS. Furthermore, the STAS but not the LSTAS of SULTR1;1 interacts weakly with OASTL111A; this interaction is only clearly detected by the sensitive leucine auxotrophy assay.

To identify the region of OASTL to which 1;2LSTAS binds, we generated a number of OASTL constructs encoding polypeptides truncated to varying extents. As shown in Fig. 1A, removing β1-β2 (the first 38 amino acids) from the OASTL (OASTL39M in Fig. 1) is enough to detect interactions in the yeast two-hybrid system. Additional truncations of OASTL at the N terminus (144M, 210S, and 175G; the latter two are not shown) did not eliminate the interaction with 1;2LSTAS, whereas an N-terminal truncation to 233S yielded a signifi-



FIGURE 2. *In vitro* interactions between OASTL and the SULTR1;2 STAS domain. *A* and *B*, *in vitro* binding assays show interaction of OASTL fused at its C terminus to His₆ (OASTL-His₆) with SULTR1;2 or SULTR1;1 STAS domain (with or without L region), which is fused to MBP at its N terminus (MBP-1;2STAS, MBP-1;1LSTAS, MBP-1;1STAS, and MBP-1;1LSTAS). An equal amount of OASTL-His₆ was co-incubated with MBP or the MBP fusion proteins at 22 °C for 1–4 h with gentle rocking; MBP or MBP fusion proteins were trapped on an amylose resin, which was washed three times with the reaction buffer (minus the proteins) prior to releasing the amylose bead-bound fractions (PPT, precipitate) and resolving the released proteins by SDS-PAGE followed by immunological detection using His₆- and MBP-specific antibodies. *C*, interaction of SULTR1;2 with OASTL. C-terminally 4MYC-tagged SULTR1;2 (SULTR1;2-4MYC) was transiently expressed in tobacco epidermal cells, and the microsomal fraction was co-incubated with N-terminally His₆-tagged OASTL (His₆-OASTL). A Ni-NTA resin was used to bind His₆-OASTL, and the unbound (*U*), the flow-through of the last wash (*W*), and the resin-bound (*B*) fractions were assayed for His₆-OASTL and SULTR1;2-4MYC by SDS-PAGE followed by immunological detection using His₆- and MYC-specific antibodies.

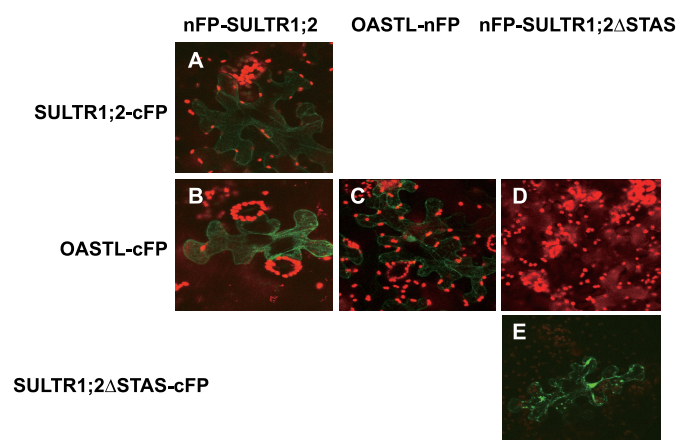


FIGURE 3. Interaction of SULTR1;2 with OASTL based on *in vivo* BiFC assay. The N- or C-terminal halves of the fluorescent protein (nFP or cFP, respectively) were fused to OASTL, or to SULTR1;2, with (SULTR1;2) and without (SULTR1;2ΔSTAS) the STAS domain. Each set of plasmids carried by *A. tumefaciens* was co-introduced into tobacco epidermal cells, and fluorescence from reconstitution of the complete fluorescent protein (green), as well as chlorophyll fluorescence (red), was observed by confocal microscopy. *A*, SULTR1;2 oligomerization. *B*, SULTR1;2 interaction with OASTL. *C*, OASTL oligomerization. *D*, interaction of SULTR1;2 deleted for the STAS with OASTL. *E*, oligomerization of SULTR1;2 deleted for the STAS domain.

cantly weaker signal (Fig. 1*B*). Furthermore, although a C-terminal truncation to 287A (144M-287A) did not reduce the signal strength, deletion of the C terminus to 233S (39M-233S) caused a marked decrease in the signal (Fig. 1*B*). These results suggest that OASTL interacts with the STAS domain in the region around amino acid 233S, which is located in a loop between β8 and β9 (19).

In Vitro Binding Assays Confirm the Interaction between the SULTR1;2 STAS Domain and OASTL—The interaction between STAS domains and OASTL was further tested by an *in vitro* binding assay using recombinant proteins expressed in *Escherichia coli*. The DNA encoding the STAS domain of SULTR1;2 and SULTR1;1, with or without the L region, was fused to the C terminus of the MBP. The resulting constructs were expressed in *E. coli* to generate fusion proteins MBP-1;

2LSTAS, MBP-1;2STAS, MBP-1;1LSTAS, and MBP-1;1STAS. OASTL was also expressed in *E. coli* but as the His₆-tagged protein OASTL-His₆. To monitor interactions *in vitro*, recombinant OASTL-His₆ was incubated with various MBP-STAS fusion proteins, and the MBP-STAS was then purified using an amylose resin; OASTL-His₆ that co-purified with MBP-STAS was detected immunologically. Binding of OASTL to MBP-1;2STAS was most readily detected when OAS was included in the binding assay (Fig. 2*A*). The STAS domain of SULTR1;2 with the L region (MBP-1;2LSTAS) also bound OASTL in the presence of OAS, whereas the binding of OASTL to the STAS or

LSTAS of SULTR1;1 was barely detectable (Fig. 2*B*). These results are congruent with those of the yeast two-hybrid assays (Fig. 1*A*). Neither 1 mM cysteine nor various SO₄²⁻ concentrations had a significant effect on OASTL-1;2STAS interaction (supplemental Fig. 1), although S²⁻ did cause a reduction in this interaction (data not shown), probably because, over the course of the assay, the added S²⁻ facilitates OASTL activity and the rapid consumption of OAS in the synthesis of cysteine. The His₆-OASTL protein also pulled down full-length SULTR1;2-4MYC expressed in *N. benthamiana* (tobacco) epidermal cells (Fig. 2*C*). The two fusion proteins were co-incubated, and the His₆-OASTL was captured on a Ni-NTA resin, which was then subjected to three stringent washes. The bound His₆-OASTL (Fig. 2*C*, lane *B*) was liberated along with a significant amount of SULTR1;2-4MYC (Fig. 2*C*, compare lanes *W* and *B*, left panel). No SULTR1;2-4MYC bound to the column in the absence of His₆-OASTL (Fig. 2*C*, see lanes *W* and *B*, right panel).

Interaction between the STAS Domain and OASTL in Vivo—Interactions between SULTR1;2 and OASTL were analyzed *in vivo* using the plant BiFC system (24). The N- or C-terminal halves of the fluorescent protein (nFP and cFP, respectively) were fused to full-length SULTR1;2, SULTR1;2 deleted for the STAS domain (SULTR1;2ΔSTAS), or full-length OASTL. Combinations of these proteins were expressed transiently in tobacco leaf epidermal cells. Fig. 3 shows the images observed by confocal microscopy 5 days after co-inoculation of the plant leaf cells with a combination of *A. tumefaciens* separately carrying genes for the indicated proteins. The fluorescence pseudo-colored in red in the images is from chlorophyll, and the green fluorescence results from close proximity of nFP and cFP that occurs when the polypeptides fused to nFP and cFP interact. As shown, SULTR1;2 proteins interact with each other (Fig. 3*A*), and this interaction does not depend on the STAS domain (Fig. 3*E*). Another SLC26A family protein, Prestin, also forms dimers/oligomers (25). Homodimerization of OASTL polypeptides, as established previously (26), is also observed (Fig. 3*C*). Furthermore, the *in vivo* assay shows a clear interaction between OASTL-cFP and nFP-SULTR1;2 (Fig. 3*B*), which is

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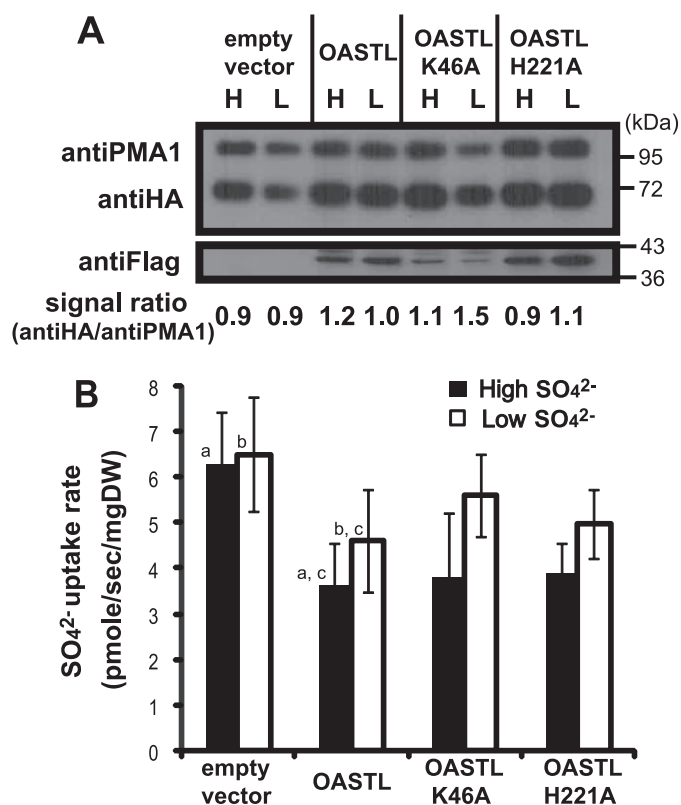


FIGURE 4. Consequences of SULTR1;2-OASTL interactions on SO_4^{2-} uptake and OASTL activity. *A*, SULTR1;2-HA expression in *S. cerevisiae* cells or in *S. cerevisiae* cells also expressing OASTL-FLAG (OASTL) or the K46A or H221A mutant of OASTL-FLAG (OASTL K46A or OASTL H221A). Signal strengths were quantified with ImageJ and presented as a ratio of anti-hemagglutinin antibodies to anti-PMA1. *B*, SO_4^{2-} uptake by SULTR1;2-HA in the various cells described in *A*. SO_4^{2-} uptake by cells grown in high (H) or low (L) SO_4^{2-} medium was assayed. Error bars are standard deviations ($n = 5-13$). Lowercase letters *a-c* indicate the statistically significant differences observed based on Student's *t* tests between vector control and OASTL co-expression tested under the same sulfur condition (*a* and *b*) or between different sulfur conditions with the same transformant (*c*); $p = 6 \times 10^{-5}$ for *a*, $p = 0.03$ for *b*, and $p = 0.056$ for *c*.

absolutely dependent on the SULTR1;2 STAS domain; removal of the STAS domain abolishes this interaction (Fig. 3D), although the SULTR1;2 Δ STAS is still synthesized, accumulates, and shows homodimerization in the BiFC system (Fig. 3E).

Activity of SULTR1;2 Expressed in Yeast Cells Is Suppressed by Co-expression of OASTL—To gain insights into the functional relevance of the OASTL-SULTR1;2 interaction, we evaluated the effects of the interaction on SO_4^{2-} transport activity in *S. cerevisiae* mutant cells null for the endogenous SO_4^{2-} transporters (14). Initial experiments demonstrated that co-expression of SULTR1;2 with OASTL in cells grown in high (1 mM) or low (10 μM) SO_4^{2-} concentrations did not significantly alter SULTR1;2 protein levels (Fig. 4A). However, yeast cells co-expressing OASTL with SULTR1;2 show a statistically significant reduction in the SO_4^{2-} uptake rate compared with cells only expressing SULTR1;2 (Fig. 4B), suggesting that the interaction of OASTL with SULTR1;2 negatively impacts transporter activity. This decrease in activity, which was an average of more than five independent experiments, using freshly transformed cells for each of these experiments, is more pronounced in cells maintained in high relative to low SO_4^{2-} medium (Fig. 4B).

To determine whether the negative effect of OASTL on SO_4^{2-} transport activity requires the catalytic activity of the enzyme, OASTL with a K46A substitution, which eliminates cysteine synthase activity (19), was co-expressed with SULTR1;2 in yeast cells; once again five separate experiments were performed. Results with the mutated OASTL were similar to those obtained with the unaltered protein (Fig. 4B). Furthermore, the lesion did not cause a significant change in the ratio of SULTR1;2 to PMA1 in the plasma membranes of the transformed lines (Fig. 4A) or disrupt interactions between SULTR1;2 LSTAS and OASTL (supplemental Fig. 2), suggesting that there is a direct effect of OASTL-SULTR1;2 interaction on SO_4^{2-} transport activity that is independent of OASTL catalytic activity.

Finally, OASTL with an H221A lesion was also tested for its effect on SULTR1;2 activity. The H221A mutation in OASTL was previously shown to inhibit its ability to interact with serine acetyltransferase (SAT) (19). However, our results show that there is no significant alteration in the OASTL(H221A)-LSTAS interaction, based on the yeast two-hybrid analysis (supplemental Fig. 2A), and that this interaction still elicits a reduction in SULTR1;2 activity (Fig. 4B).

Cysteine Synthase Activity of OASTL Is Enhanced by Co-incubation with 1;2STAS—We also developed an *in vitro* assay to evaluate the effect of 1;2(L)STAS-OASTL interaction on OASTL activity. Purified OASTL-His₆ was co-incubated with no protein partners in 1 \times phosphate-buffered saline (mock), MBP, MBP-1;2STAS, MBP-1;2LSTAS, MBP-1;1STAS, or MBP-1;1LSTAS at 22 $^{\circ}\text{C}$ for 30 min prior to determining cysteine synthase activity. The results suggest that 1;2STAS, but not 1;1STAS, enhances OASTL activity (Fig. 5A). Similar results were obtained with three independently purified protein preparations. Because the efficiency of *in vitro* binding is not high (Fig. 2, A–C) and protein interaction is probably only transient, an equimolar concentration of 1;2STAS and OASTL does not result in significant OASTL activation. However, OASTL activity increased by a factor of 2.0 when a 12-fold molar excess of the STAS domain was used in the assay. The LSTAS domains of both SULTR1;2 and SULTR1;1 were also tested, and surprisingly neither had a significant effect (Fig. 5). When the L region of the LSTAS polypeptide is connected to MBP rather than to the catalytic domain of the transporter, it may be more mobile (attachment to the catalytic domain may restrict mobility in specific ways), which could markedly affect interactions with partner proteins. More information is required to develop a better understanding of this aspect of the work.

DISCUSSION

The levels of many transport proteins are subject to regulation by ubiquitination and complex signals that influence endocytic sorting (27–30). Indeed, both the levels and activities of transport proteins may be regulated by protein modifications and interactions, including phosphorylation (31–33). Here, we report a novel example in which transporter activity may be regulated by interactions with an enzyme involved in assimilation of the substrate of the transporter. SULTR1;2 transports SO_4^{2-} into roots of *A. thaliana* (6). Both *in vivo* and *in vitro*

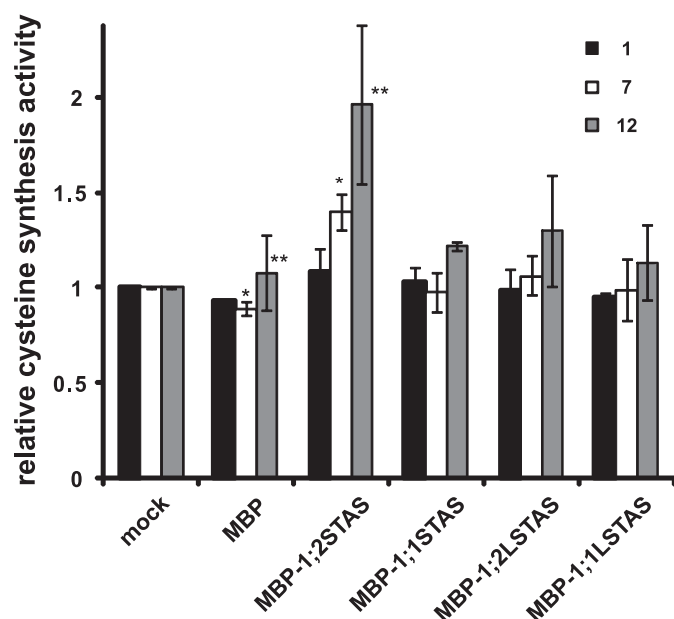


FIGURE 5. **Effect of STAS domain on OASTL activity.** OASTL activity was measured after co-incubation with SULTR1;2STAS, SULTR1;2LSTAS, SULTR1;1STAS, and SULTR1;1LSTAS, all fused to MBP (MBP-1;2STAS, MBP-1;2LSTAS, MBP-1;1STAS, and MBP-1;1LSTAS), as well as with free MBP. OASTL was added at 0.5 ng/ μ l, and the STAS-MBP fusions were added to the reaction mixtures at the indicated molar ratios (OASTL:MBP molar ratio were 1:1, 1:7, 1:12, as shown in black, unfilled, and gray bars, respectively). Asterisks demarcate values judged to be significantly different based on Student's *t* tests: *, $p = 0.002$; **, $p = 0.005$ ($n = 3-5$).

assays demonstrate that this transporter interacts with OASTL, the enzyme involved in bonding S^{2-} to OAS to form cysteine.

Interaction through the STAS Domain

As demonstrated by the yeast two-hybrid results, the STAS domain of SULTR1;2 is essential for the interaction between SULTR1;2 and OASTL. Using the BiFC technique in tobacco cells, we demonstrated analogous interactions *in planta*. The interaction between SULTR1;2 and OASTL based on BiFC analysis was shown to depend on the STAS domain (Fig. 3). A truncated SULTR1;2 polypeptide, deleted for most of the STAS domain, accumulates in cells, as demonstrated by the positive signal from homomeric interaction analysis (interaction between catalytic domains of SULTR1;2, see Fig. 3E), but shows no interaction with OASTL (Fig. 3D). A more extensive C-terminal truncation of SULTR1;2 was previously shown to destabilize the transporter protein (8).

Region in OASTL Involved in the Interaction

The loop located between $\beta 8$ and $\beta 9$ appears to be the site on OASTL that interacts with the STAS domain (Fig. 1B). This loop is also essential for SAT-OASTL interaction. Although an H221A substitution in this loop disrupted SAT-OASTL interaction (19), it did not appear to alter the interaction between the STAS domain and OASTL (supplemental Fig. 2). Furthermore, it did not eliminate suppression of transport activity elicited by SULTR1;2-OASTL interaction (Fig. 4B). These results raise the possibility that the STAS domain competes for SAT binding to OASTL at the loop between $\beta 8$ and $\beta 9$ but that this binding does not require His-221.

Interaction Is Enhanced by OAS

The *in vitro* binding of the STAS domain to OASTL was significantly promoted when OAS was added to the reaction, although the interaction was observed even without OAS (Fig. 2A). The concentration of OAS needed to generate the most pronounced interaction is ~ 2 mM, which is high considering that the OAS concentration in root cells of sulfur-starved plants is reported to be 60 nmol/g fresh weight for *A. thaliana* (34) and 200 nmol/g fresh weight for potato (35). The reason that OAS may need to be added to the assay at a relatively high concentration to observe an effect may reflect a number of factors. The OAS concentrations that have been reported are from bulk measurements in disrupted tissue; local concentrations may be much higher, especially in the proximity of the enzyme SAT, which synthesizes OAS. Furthermore, OAS is not stable and is rapidly converted to *N*-acetylserine *in vitro* (36). Thus, the amount of OAS in the reaction mixture may decline significantly over the course of the assay.

Functional Relevance of the Interaction

There are only a few other instances that have been reported in which a soluble protein in plants interacts with an integral membrane protein transporter. The SOS1 protein, a plasma membrane-localized Na^+/H^+ antiporter, prevents Na^+ toxicity by facilitating its extrusion from cells, and under oxidative stress conditions, the C terminus of the transporter interacts with the soluble RCD1 protein, a regulator of oxidative stress responses (37). However, the precise functional and regulatory consequences of this interaction are not known.

SULTR1;2 Activity and Interaction—We demonstrated that co-expression of OASTL with SULTR1;2 in yeast cells decreased SO_4^{2-} transport activity of SULTR1;2 (Fig. 4). There are a number of ways in which the transport of SO_4^{2-} into cells may be regulated. These ways would include modulation of the rate of substrate translocation or alteration in the number of transporter molecules that are present at their site of function. The total accumulation of SULTR1;2 in yeast cells expressing OASTL, based on immunological analyses, does not appear to be different in the presence or absence of OASTL. These results suggest that the stability and biogenesis of SULTR1;2 is similar in transformants expressing only SULTR1;2 and those expressing both OASTL and SULTR1;2, although we do not know if the level of the transporter at the site of function on the plasma membrane is the same in the two transformants. SULTR1;2 may also interact with cysteine, and elevated intracellular cysteine levels may retard transport. Because a high cellular cysteine concentration can be toxic to cells (38), this type of regulation would likely require the generation of a cysteine-rich microenvironment in the neighborhood of the SO_4^{2-} transporters. Such a situation could occur as a consequence of interaction of OASTL with SULTR1;2; cysteine would be synthesized at the site of SO_4^{2-} transport. However, based on our results, there appears to be a direct effect of OASTL-SULTR1;2 interaction on SO_4^{2-} transport activity rather than an effect of the cysteine synthesized by OASTL. This conclusion is based on the finding that OASTL with a K46A mutation, which was previously shown to be catalytically inactive (19), can still bind

Interaction of Sulfate Transporter with Cysteine Synthase

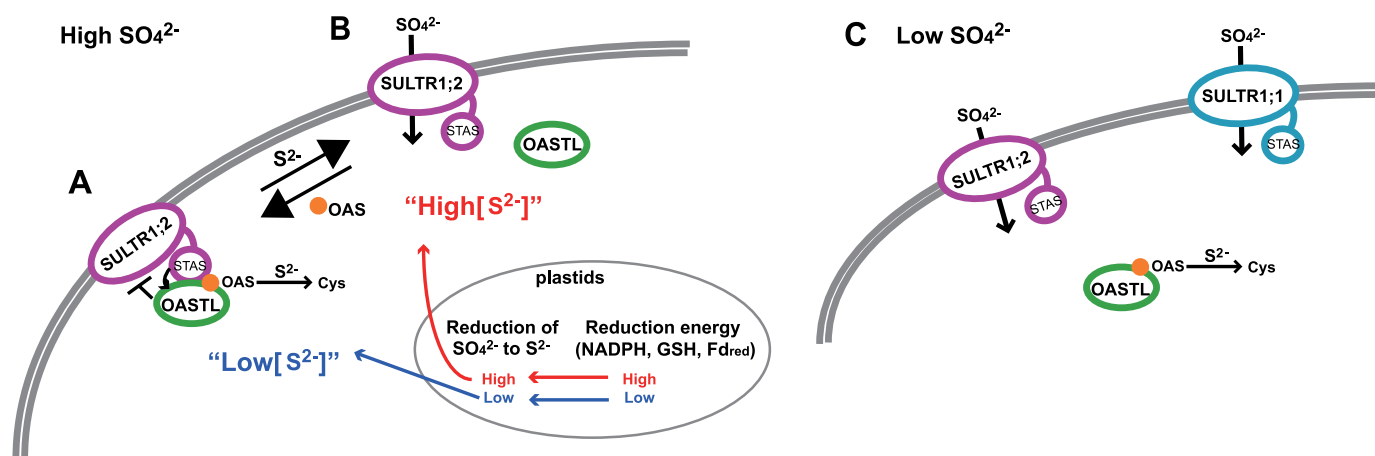


FIGURE 6. Model depicting potential regulatory function of SULTR1;2-OASTL interaction. *A* and *B*, high SO_4^{2-} condition. A decline in cellular energy status compromises the ability of cells to reduce SO_4^{2-} in plastids and/or mitochondria, which favors low S^{2-} and high OAS in the cytosol (in a high SO_4^{2-} environment). Elevated OAS leads to STAS-OASTL interaction. The interaction may negatively affect SULTR1;2 activity and positively affect OASTL activity; OASTL remains active when free in the cytoplasm, has increased activity when bound to SULTR1;2, and becomes inactive when complexed with SAT. *B*, if S^{2-} synthesis keeps pace with OAS production, the OAS is consumed in the formation of cysteine, OASTL is released from the STAS domain, and SULTR1;2 is free to transport SO_4^{2-} . Upon re-binding of OAS to OASTL, the situation depicted in *A* is re-established. Repeated cycling between (*A* and *B*) coordinates SULTR1;2 activity with the ability of the cell to reduce SO_4^{2-} . *C*, low SO_4^{2-} condition. When SO_4^{2-} is low, a conformational change in SULTR1;2 may lessen the STAS-OASTL interaction, allowing higher rates of SO_4^{2-} transport activity despite potentially high OAS levels. Furthermore, low SO_4^{2-} conditions favor the synthesis of SULTR1;1, a high affinity transporter that is insensitive to OASTL regulation. *Fdred*, reduced ferredoxin; *GSH*, glutathione.

SULTR1;2 and negatively impact SO_4^{2-} transport activity in yeast cells. However, even the wild-type enzyme may not have high activity in yeast as yeast does not synthesize high levels of OAS (39). Interestingly, the addition of external OAS prior to the *in vivo* assay does not modify uptake activity, possibly because supplemental OAS is rapidly metabolized by the cells or because endogenous levels of OAS or *O*-acetylhomoserine are already sufficient to saturate the repressive interaction.

The mode by which the OASTL-SULTR1;2 interaction alters SO_4^{2-} transport activity is not known, but it is possible that direct contact between the proteins causes a change in the SULTR1;2 conformation that results in decreased transport rates. It is also possible that the altered activity is an indirect consequence of the interaction. For example, OASTL-SULTR1;2 interaction might influence the binding of other proteins that catalyze post-translational modifications.

OASTL Activity—The interaction between OASTL and SULTR1;2 appeared to cause increased OASTL activity, an effect specific for the SULTR1;2 STAS domain; no change in activity was observed when the SULTR1;1 STAS domain was used in the assay (Fig. 5). Because the efficiency of *in vitro* binding is generally not high (Fig. 2), and the interaction between the proteins is probably only transient, an equimolar addition of the SULTR1;2 STAS domain and OASTL is not sufficient for activating OASTL. However, an ~2-fold activation was evident when the STAS domain was added to the assay at a 12-fold molar excess relative to OASTL. Interestingly, when the L region was maintained at the N terminus of the STAS domain, the OASTL activity enhancing effect of the STAS domain was diminished. When the L region is present on the LSTAS polypeptide but not connected to the catalytic domain of the transporter, it may be highly mobile; attachment to the catalytic domain would likely restrict LSTAS domain mobility, which could strongly affect its interactions with partner proteins.

Model—Vascular plants have a number of OASTL and SAT proteins that differentially localize to plastids, mitochondria,

and the cytosol. Although the mitochondrial OASTL/SAT was shown to be most important for cysteine synthesis (40–42), the cytosolic isoforms may be regulatory. Based on the results reported here, we propose a model for the regulation of SO_4^{2-} assimilation by roots that incorporates the consequences of the interaction between SULTR1;2 and the root cytosolic OASTL (Fig. 6). At high SO_4^{2-} concentrations, when SULTR1;2 is the dominant transporter responsible for SO_4^{2-} uptake from the soil, direct interaction of SULTR1;2 and OASTL helps coordinate SO_4^{2-} transport with S^{2-} production and cysteine synthesis. Availability of S^{2-} in the cytoplasm of the root cells would be a major control feature of this model.

OASTL would associate with the STAS domain of SULTR1;2 when OAS rises, and this interaction inhibits transporter activity (Fig. 6A). This relationship may seem counter-intuitive as OAS has been suggested to be a positive regulator of SO_4^{2-} assimilation, at least at the transcriptional level, because OAS accumulation is indicative of diminished sulfur levels (34). However, OAS can also be temporarily and locally in excess when the supply of S^{2-} , and not the supply of SO_4^{2-} , is limited, which may be a relatively common situation in the environment. Most S^{2-} is derived from SO_4^{2-} reduction in root plastids and catabolism of organic sulfur-containing compounds. SO_4^{2-} reduction requires ATP and reducing equivalents that are generated by photosynthetic electron transport and respiration; thus, the reduction of SO_4^{2-} in the root would depend on the level of carbon fixation in leaves and the transport of fixed carbon to roots, where it can be used for reductive assimilation of SO_4^{2-} . Therefore, even when SO_4^{2-} is abundant in the environment, if metabolic activity and the generation of reducing power are low, which might occur under conditions where photosynthetic activity slows or stops, the cytosolic S^{2-} concentration would decline leading to accumulation of OAS. Under such circumstances, it would be advantageous to repress transporter-dependent SO_4^{2-} uptake activity as it is an energy-consuming process, and even if the anion were taken into the

cell, reductive assimilation would be limited. An elevated level of OAS combined with reduced S^{2-} availability would favor sustained binding of OAS to OASTL, which in turn would promote the interaction of "OAS-loaded" OASTL with SULTR1;2. This interaction would limit SULTR1;2 activity, despite high environmental SO_4^{2-} levels. As OAS is consumed in the synthesis of cysteine, under conditions where S^{2-} production begins to keep pace with OAS generation, OASTL would dissociate from SULTR1;2 releasing the transporter from inhibition (Fig. 6B). Repeated cycles of binding and release may help maintain a level of SO_4^{2-} transport that is coordinated with the capabilities of the plant to generate S^{2-} and cysteine, as governed by intracellular reductant and energy levels.

The model presented above would also integrate with our understanding of the control of the cysteine synthase complex, which is composed of SAT and OASTL. Various transgenic experiments have shown that SAT, and not OASTL, catalyzes the limiting reaction in the synthesis of cysteine (35, 43–46). The level of OAS appears to modulate cysteine synthase complex activity and the generation of OAS; high OAS levels cause dissociation of the complex, and the free SAT tends to aggregate and exhibit a lower affinity for its substrates. In contrast, the dissociated OASTL has elevated activity because its affinity for OAS and S^{2-} increases (47–49). Also, SAT activity and OAS levels may be modulated by L-cysteine feedback inhibition (50). In the model that we propose, as the OAS levels rise locally in the cells, the cysteine synthase complex would dissociate and SAT activity would decline as OASTL activity increases. Indeed, the activity of OASTL may be further stimulated because it would bind OAS and interact with SULTR1;2, an interaction that also inhibits the transport of SO_4^{2-} into the root cells. However, because the cellular level of OASTL is high (51), the regulatory function of the SULTR1;2-OASTL interaction is likely to have a greater impact on SULTR1;2-dependent SO_4^{2-} transport than on overall OASTL activity and cysteine biosynthesis.

The impact of the regulatory mechanism proposed above during sulfur deprivation (Fig. 6C), when the organism would require efficient acquisition and utilization of both external and internal sulfur resources, would not be severe for two reasons as follows: (i) inhibition of SULTR1;2 activity as a consequence of OASTL binding is less under sulfur-depleted than under sulfur-replete conditions (Fig. 4B) as suggested from the SO_4^{2-} transport activity assay in yeast cells, and (ii) there is a second transporter that is synthesized in *A. thaliana* roots in response to sulfur deprivation, SULTR1;1, that becomes the major SO_4^{2-} transporter in root cells of sulfur-deprived plants, and this transporter protein does not appear to interact with OASTL nearly as strongly as SULTR1;2 (Figs. 1 and 2B). Although other cytosolic OASTL isoforms are present in *A. thaliana* (40), the potential for these isoforms to interact with sulfate transporters and modulate their activities remains to be explored.

In sum, we describe a potential novel regulatory mechanism controlling SO_4^{2-} transport into root cells that involves OAS/ S^{2-} -modulated interaction of OASTL with SULTR1;2. Similar regulatory mechanisms may exist for other nutrient transporters, and the proposed model may be one example of a more

general mode by which cells coordinate activities of metabolic pathways with physically distinct transport processes.

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